MATING TYPES IN TETRAHYMENA [1]

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The potentialities of *Tetrahymena* as a genetic tool have long been considered by those working with this ciliated protozoan. Its value stems from the fact that it is one of the few animal cells that can be grown in a defined medium. The failure of laboratory strains to reproduce sexually has been the chief obstacle to its use in genetic studies. The recent discovery of conjugation (selfing) in several strains (AA) taken from the Ann Arbor area (Elliott and Nanney, 1952) stimulated a further search for wild strains in which mating types might be found. These have been found and the purpose of this paper is to describe certain aspects of their physiology and cytology, and to discuss how they may be handled in the laboratory with a view to their use in genetic studies.

Materials and Methods

One hundred twenty-seven clones of *Tetrahymena* sp. (probably *T. pyriformis*) were established from 15 different fresh water habitats in the Woods Hole, Massachusetts area (Elliott and Gruchy, 1952). Three of these turned out to be selfers whereas all others did not conjugate within the clone. Cross-matching in all combinations finally yielded a pair of mating clones which came from one pond. Sixteen of the 17 other clones from this source mated readily with either one or the other of these two. Clone 52 from another pond conjugated with both of the first two. These three have been designated WH (Woods Hole) strains 6, 14, and 52, mating types I, II, and III, respectively. Throughout this paper strains 6, 14, and 52 will be referred to only as mating types I, II, and III. Two strains which will mate with types I, II, and III have also been isolated from a lake in northern Minnesota (Park Rapids area). One of these is another mating type I which conjugates with types II and III; the other, a type II, mates with types I and III.

All clones were established in axenic cultures by placing several cells from a bacterized culture (0.1% Cerophyll seeded with *Aerobacter aerogenes*) in a depression slide containing a mixture of penicillin G and streptomycin (250 γ/ml. of each) in the stock medium. The stock medium contains Bacto-tryptone 5 gm., Bacto-proteose-peptone 5 gm., sodium acetate 1 gm., thiamine HCl 0.002 gm., yeast extract 0.1 gm., KH_2PO_4 1 gm., and 1000 ml. of glass-distilled water. Its pH is adjusted to 7.2 with NaOH. After approximately 12 hours, single-cell isolations were made into several depressions containing the antibiotic medium. Twenty-

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four hours later, during which time growth of the protozoan had occurred (about 10 divisions), the entire drop was transferred to a test tube containing the stock medium without the antibiotics. Only rarely did this procedure fail to eliminate contaminating organisms as verified by the customary sterility tests.

Stock lines have been maintained continuously in this medium and, except where otherwise stated, all experiments are conducted with organisms grown in this medium. Loop inoculations reach peak growth in about 7 days and the cells remain viable for two or more months, thus permitting the handling of large numbers of clones without frequent sub-culturing.

Experimental

I. Nutrition

In determining the nutritional requirements of the mating types, the eleven amino acids and seven B-vitamins essential for the long-established strain E (Elliott, 1949, 1950) were tested by single omissions from the complete defined medium that supports normal growth of strain E (Table I). The results of this

Table I

Defined medium

	Micrograms per milliliter
L-Arginine · HCl	150
L-Histidine · HCl · H_2O	110
DL-Isoleucine	100
DL-Leucine	140
L-Lysine. · HCl · H_2O	70
DL-Methionine	70
DL-Phenylalanine	100
DL-Serine	180
DL-Threonine	180
L-Tryptophan	120
DL-Valine	60
Asparagine	85
Dextrose	1000
Sodium Acetate	1000
$MgSO_4 \cdot 7H_2O$	10.0
K_2HPO_4	100.0
$Zn(NO_3)_2 \cdot 6H_2O$	5.0
$FeSO_4 \cdot 7H_2O$	0.5
$CuCl_2 \cdot 2H_2O$	0.5
Uracil	25.0
Cytidylic acid	25.0
Guanylic acid	25.0
Adenylic acid	25.0
Ca pantothenate	0.10
Nicotinamide	0.10
Pyridoxine HCl	2.00
Riboflavin	0.10
Folic acid	0.01
Thiamine HCl	1.00
Protogen or Thioctic acid	1 unit

series of experiments indicate no nutritional differences between the mating types (I, II, III) and strain E insofar as these nutrilites are concerned. In the light of experience with other strains it is unlikely that differences in purine, pyrimidine, and inorganic requirements would be found if sought for.

A comparative growth study of the mating types and strain E in defined medium showed remarkable similarity among the three mating types (Fig. 1). Strain E, perhaps as a result of its long maintenance in test tubes (20 years), grew faster after the third day and reached a higher maximum.

II. General morphology

Mating types I, II, and III have been identified as *Tetrahymena pyriformis* by Corliss (personal communication). There are no apparent morphological differences among the three types. All possess a single macronucleus and at least one micronucleus. The number of micronuclei varies within a single clone; for example, in one series of counts of the Type I clone the number varied as follows: 1 micronucleus, 78%; 2 micronuclei, 16%; 3 micronuclei, 3%; and 4 micronuclei, 2%. A similar range was found in Type II clones. An occasional individual without a micronucleus (about 1%) can be found in laboratory cultures but no amicronucleate clones have been established from nature.

The presence of more than one micronucleus complicates the cytological picture when the steps in conjugation are traced. Since unimicronuclear clones were desirable for cytogenetic studies of conjugation an effort was made to control the number of micronuclei within a clone.

Seventeen clones of mating types I and II were established and after 2–5 days of incubation were stained and the micronuclei counted. One hundred individuals from each clone were examined for their micronuclear number. Of the 17 clones, 7 were found to be as variable as the original stocks; 6 proved to be consistent for bimicronucleate individuals; and 4 contained only unimicronucleate organisms. These 4 clones with single micronuclei were carried through three serial transfers and, after two weeks, stained preparations were made from the last transfers None of these clones had remained constant for single micronuclei as indicated by average counts. However, no exconjugants with more than one micronucleus have been observed and, since multimicronucleate conjugants occur regularly, one of the results of the sexual process is to establish uniformity in the micronuclear number for the species.

The maintenance of a constant number of micronuclei in clone cultures seems to depend on chance alone. The presence of multi-micronucleate cells may be correlated with the formation of doublets (two-mouthed individuals) as suggested by Corliss (personal communication).

The possibility that the number of micronuclei might be influenced in some way by age of the culture was tested by staining 2-, 8-, and 40-day old cultures for examination. Data derived from such preparations gave no indication that the number of micronuclei was correlated with age.

During the first prezygotic division of conjugation, chromosomes (or chromosome aggregates) become clearly visible. There are four (or possibly five) rela-

tively large, thick rods which presumably represent the haploid number. Because these "chromosomes" are comparatively large and so few in number when compared to most other ciliates they provide excellent material for detailed cytological studies.

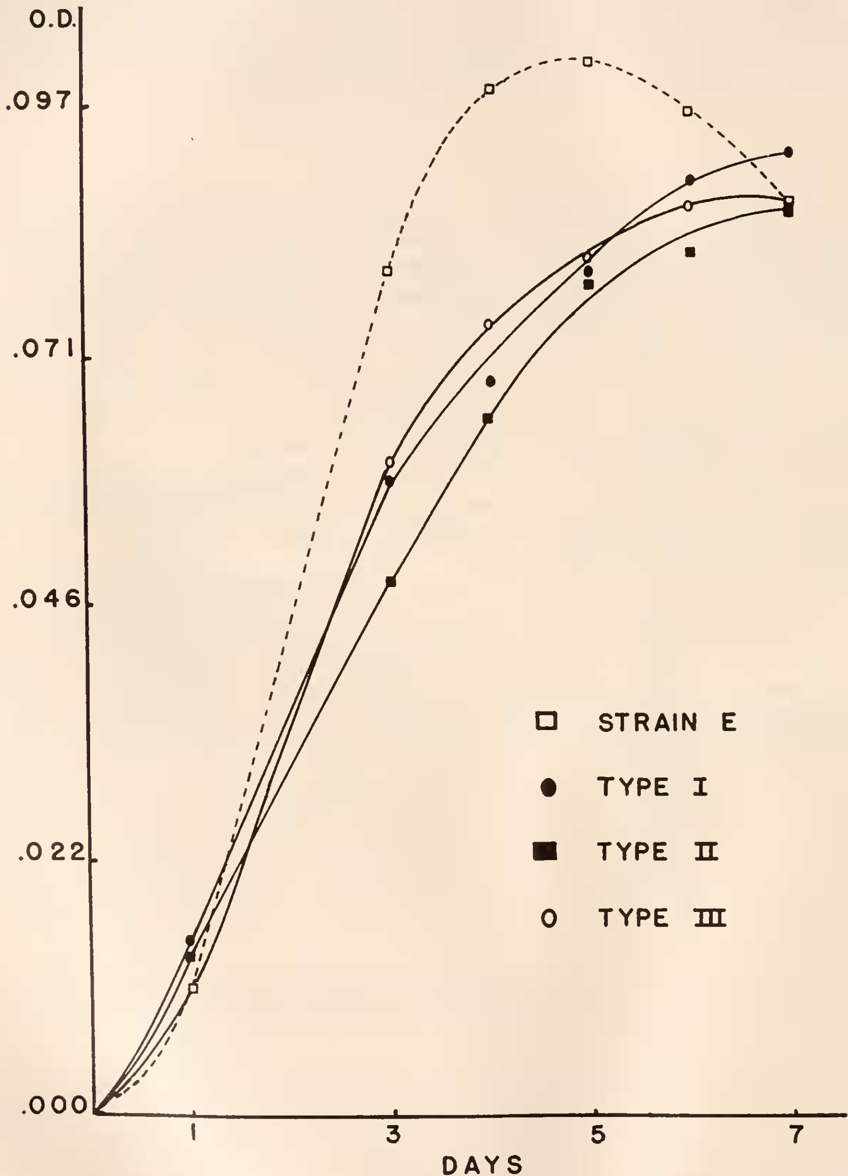


FIGURE 1. Comparative growth of strain E and the three mating types in defined medium. Growth as measured by optical density (O.D.) is plotted against time in days.

III. Conjugation

When washed cells of opposite mating types are mixed under appropriate conditions conjugation takes place. In order to rule out the possibility of selfing, several checks were made. By mixing a small number of cells (15–20) of one mating type with a great many (500 or more) of the opposite mating type and counting the number of pairs that appear as compared to the control in which equal numbers of cells were mixed, it was possible to prove conclusively that selfing did not occur. Furthermore, selfing has never been seen when the mating types are subjected to the same conditions that initiate the process in our selfing (AA) strains.

The preliminary mating reactions reported for some other ciliates, *i.e.*, agglutination and the formation of large clumps of animals, are absent in *Tetrahymena*. Pairs of cells are formed directly. Two animals become attached at their preoral surfaces near their anterior ends with bodies flaring at a wide angle. They are loosely attached at first and often twist and break apart. The attachment soon becomes quite firm. A conjugating pair remains active and swims as a unit with a characteristic spiraling motion.

The following description is intended to cover the normal process of conjugation in *Tetrahymena pyriformis* as seen in mating types I and II. Observations on matings between I and III, and II and III show no differences greater than the normal variation found when mating I and II. The general features of conjugation resemble those reported by Maupas (1889) for *Tetrahymena* [*Leucophrys*] *patula* (Corliss, 1953), and recently by Nanney (1953) for the selfing AA (Ann Arbor) strains of *Tetrahymena pyriformis*. In certain details the phenomenon strikingly resembles the description of autogamy in *Tetrahymena rostrata* as given by Corliss (1952a). Abnormalities, such as the occurrence of multimicronucleate conjugants and triples in conjugation, are ignored in the present paper. Also, no attempt has yet been made to work out the exact time relationships of the sequence of stages in conjugation.

A. Cytology. Cytological studies were made from material fixed in hot Schaudinn's solution and stained according to Dippell and Chao's modification of the DeLameter stain (Sonneborn, 1950). A fast green counterstain was applied in most cases. Fixed material was processed in bulk in centrifuge tubes.

The following stages are indicated diagrammatically in Figure 2. Photomicrographs of some of the stages appear in Plate I. During conjugation two cells of opposite mating types are attached only at their oral surfaces near their anterior ends.

In preconjugants and in recently attached conjugants the single micronucleus is located near the macronucleus (Stage 1). Soon after the conjugants come together the micronucleus elongates into a curved, threadlike "crescent" stage of the first prezygotic division (Stage 2). This crescent later shortens, chromosomes become visible, and the division is completed, resulting in two daughter nuclei in each cell (Stage 3). Each daughter nucleus divides again in the second prezygotic division, producing four nuclei (Stage 4). It is assumed that one of the first two divisions is reductional, as is the case in other ciliates, and that the resulting four nuclei are haploid.

Of the four nuclear products in each conjugant, only one functions in the remainder of the process. The other three eventually degenerate and disappear. The functional haploid nucleus comes in contact with the cell membrane between the conjugants where it undergoes a third prezygotic division to yield two gametic nuclei

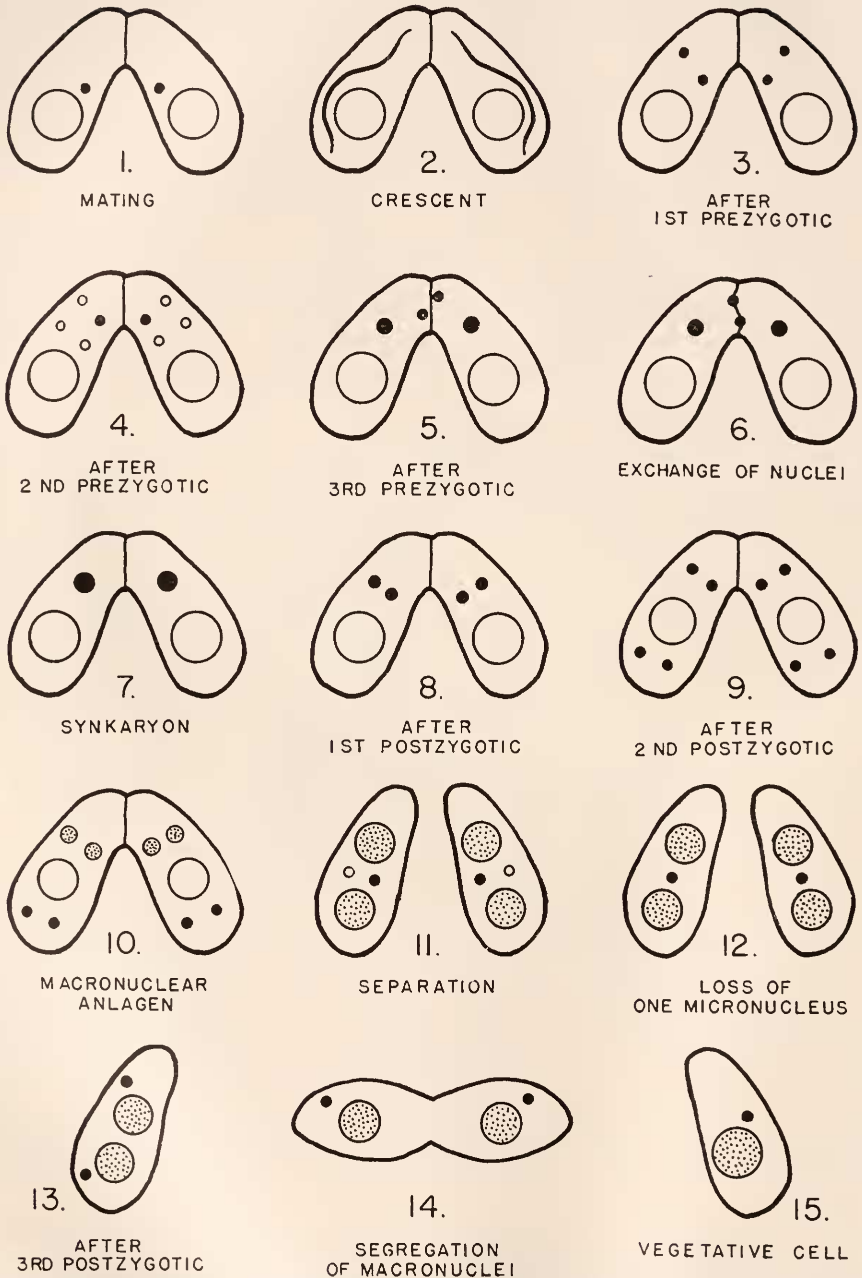


FIGURE 2. Schematic figures showing representative stages in normal conjugation of the WH strains.

(Stage 5). One of these (the stationary nucleus) takes a position anterior to the macronucleus. The other (the migratory nucleus) remains on the membrane.

In the fertilization process the migratory nucleus from each conjugant passes through the cell membrane and fuses with the stationary nucleus of the opposite cell to form the synkaryon (Stages 6 and 7).

The synkaryon then undergoes two postzygotic divisions to produce four daughter nuclei in each conjugant (Stages 8 and 9). Two of these new nuclei are located in the anterior region of the cell, and two in the extreme posterior with the macronucleus lying between. The two anterior nuclei gradually enlarge to become macronuclear anlagen which later develop into two new functional macronuclei (Stage 10). The two posterior nuclei remain small and become new micronuclei.

At about this time the conjugants separate and the old macronucleus in each exconjugant becomes smaller, more intensely stained, and spherical, eventually disappearing without fragmentation. The two anlagen become large new macronuclei and the two new micronuclei usually come to lie in the center of the cell between the macronuclei (Stage 11). One of these micronuclei degenerates, the other undergoes a third postzygotic division (Stages 12 and 13).

Each exconjugant then goes through a single fission, segregating one new micronucleus and one new macronucleus to each daughter cell (Stage 14). This fission completes the sexual process. From the original pair of conjugants four daughter cells have been produced, each of which has been restored to the normal vegetative condition with one macronucleus and one micronucleus (Stage 15).

B. Establishment of exconjugant clones. The usefulness of *Tetrahymena* as a genetic tool requires that the investigator be able to perform easily and dependably the many cross-matings involved in any genetic study with sexually reproducing laboratory animals. He must be able to mate parental types, isolate and culture exconjugant clones (F_1 generations), and then interbreed these to obtain F_2 and succeeding generations. Part of this has already been accomplished and preliminary investigations indicate that other required matings can also be made successfully.

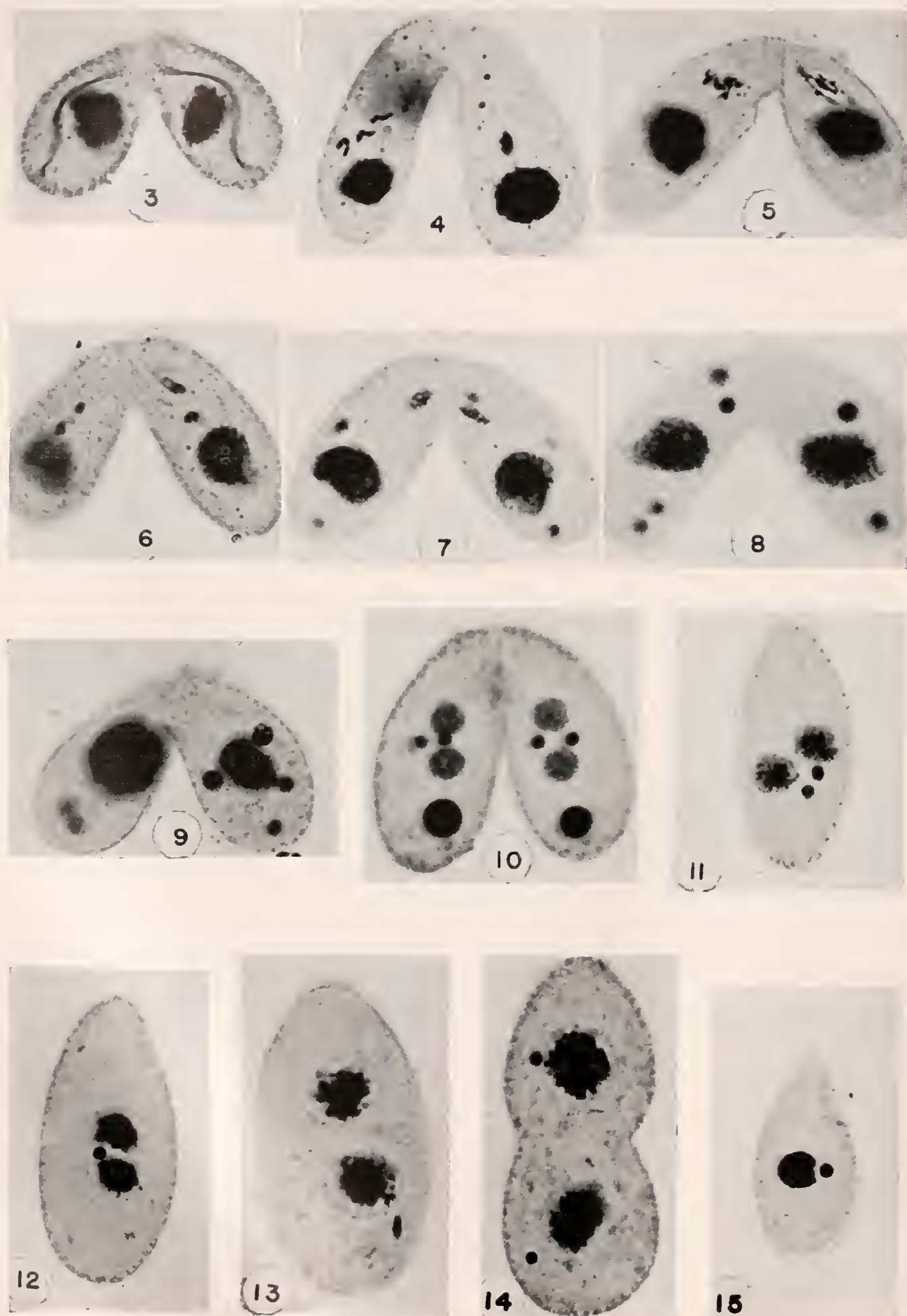
A routine procedure for obtaining exconjugant clones has been established. Since only axenic cultures of *Tetrahymena* are employed, sterile glassware and aseptic techniques are used throughout. Actively growing, young (2–7 day) stock cultures of known mating types are centrifuged and washed four times in sterile distilled water. Washed cells of two opposite mating types are mixed in shallow depression slides and allowed to stand undisturbed in distilled water for 12–24 hours. Examination under a low power microscope at intervals during this period shows the progress of pairing. It has also been found desirable to check periodically on the nuclear status of the conjugating animals. This is done quickly by staining a small sample of the cell mixture with acetocarmine and examining the wet mount under a compound microscope.

When these examinations show that many or most of the cells are paired and that conjugation is well under way, as revealed by the presence of macronuclear anlagen in many conjugants, individual pairs are isolated from the mixture with a micropipette and transferred to drops of distilled water in depression slides. These isolated pairs are then allowed to stand until the conjugants complete the sexual process and separate.

The resulting exconjugants are then picked up singly and transferred to drops of culture medium in depression slides. Within 48 hours each isolate will usually have

Plate I

Photomicrographs of stages in conjugation of *Tetrahymena pyriformis,* mating types I and II.



divided 4–8 times and the daughter cells are then transferred *en masse* to a tube of medium to establish an exconjugant clone. This clone is maintained thereafter by routine serial transfers at weekly (or longer) intervals.

Mention was made earlier of the desirability of waiting until conjugation was nearly completed before isolating conjugating pairs. It has been found that in most cell mixtures there is a certain amount of abortive conjugation, that is, of individuals coming together but separating after a time without actually conjugating. In some cases this premature separation can probably be blamed on mechanical agitation. Conjugants are only loosely attached at first and if picked up too soon are broken apart in the pipetting process. However, there seem to be other, as yet unknown, factors involved. For no apparent reason some mixtures yield a higher percentage of exconjugants than others. For example, from 154 isolated pairs 26 exconjugant cultures (17%) were obtained. From the separate groups of 20–30 isolates making up the larger series, yields of successful exconjugant cultures varied from 0% to 38%. Some of the failures can definitely be attributed to improper handling such as pipetting too early before conjugants are firmly attached and to a failure to recover isolates. Other failures, however, seem to be due to spontaneous separation of paired individuals.

Because of this high percentage of false conjugation it has been found necessary to verify each "exconjugant" by other tests. One method used is to demonstrate new macronuclei and new micronuclei in one separated individual from each isolated pair. Acetocarmine staining clearly brings out the nuclear picture. However, it is laborious and time-consuming to isolate, stain, and locate on the slide a single organism. An easier test, and one which has proved to be just as valid, is based on the fact that exconjugant clones go through a period of sexual immaturity, that is, a period during which they show no mating reaction (see below). In practice, then,

FIGURE 3. First prezygotic division showing micronuclei elongated into characteristic crescents (Stage 2).

FIGURE 4. First prezygotic division in which the crescents have condensed revealing three distinct "chromosomes." At least one other "chromosome" does not show in this photograph. These are presumably tetrads.

FIGURE 5. A stage slightly later than that shown in Figure 4, in which the "chromosomes" have separated and are moving to the poles in anaphase.

FIGURE 6. After first prezygotic division. One of the nuclei in the right conjugant is entering the second prezygotic division (Stage 3).

FIGURE 7. Four nuclear products of the second prezygotic division. Three relic nuclei are degenerating and one functional nucleus in each conjugant is undergoing the third prezygotic division (Stage 4).

FIGURE 8. Four nuclei resulting from the second postzygotic division can be seen in the left cell (Stage 9). This second division has not yet occurred in the right cell.

FIGURE 9. The two anterior nuclei in the right cell are enlarging to become macronuclear anlagen (Stage 10).

FIGURE 10. A later stage which shows the old macronuclei degenerating.

FIGURE 11. A recently separated exconjugant (Stage 11).

FIGURE 12. A later exconjugant stage in which one micronucleus has disappeared (Stage 12).

FIGURE 13. The functional micronucleus is undergoing the third postzygotic division. The exconjugant is now larger due to feeding.

FIGURE 14. Division of the micronucleus is complete and cytoplasmic cleavage is well underway, segregating a new macronucleus to each daughter cell (Stage 14).

FIGURE 15. Normal vegetative cell (Stage 15).

all newly established clones derived from "exconjugants" are mixed with mating types I, II, and III. If conjugation occurs, the clone is discarded. If conjugation does not take place the clone is considered a true exconjugant.

C. *Factors influencing conjugation*

The primary requirement for demonstrating conjugation in *Tetrahymena* is, of course, the mixing of organisms of two appropriate mating types. However, there are other factors involved which influence the mating process. As shown in the following experiments the conditions under which the cells are mixed can determine the rate at which conjugation takes place and whether or not it will occur at all.

1. Starvation. Conjugation has never been observed in the presence of nutrients, even in old cultures. However, mating can be regularly initiated by washing, which induces rapid starvation. Cells taken from any phase of the growth cycle, or those that have passed the peak growth (one to four weeks old), and washed in distilled water by centrifugation initiate conjugation at rates which are, within limits, proportional to the number of washings. The period between mixing and the appearance of the first conjugating pairs is here termed the *refractory period,* which is approximately 8 hours at room temperatures (20–22° C.) after washing once in distilled water. The refractory period is decreased to 6 hours when the cells are washed twice. Four washings reduce the time to approximately 4 hours and any more washings fail to decrease this time any further.

Additional evidence that conjugation occurs only between starved animals was obtained in the following way. Washed cells (4 times) were allowed to stand in distilled water for varying lengths of time. These starved cells decreased the refractory period at a rate which depended, within limits, on the length of time they remained in the water before mixing. For example, cells permitted to stand 24 hours in water mated in 1½ to 3 hours whereas those that starved 48 hours mated in 1–2 hours. Apparently, freshly washed cells retain sufficient reserve foods to delay mating.

Direct observation of the protozoa tends to verify this starvation requirement for mating. There is usually a definite and easily recognizable difference in appearance between cells freshly washed and those "ready" to mate. The former are large, vacuolated, and relatively sluggish whereas the starved cells, which are about to conjugate, are smaller, slender, devoid of vacuoles, and quite active.

2. Temperature. Temperature also plays an important part in the mating reaction. Several experiments were performed in an attempt to determine the optimum temperature for conjugation. Depression slides containing mixed washed suspensions of types I and II were incubated at different temperatures and examined at frequent intervals to detect the onset of conjugation. It was found, as shown in Table II, that conjugation began sooner at about 38° C. than at lower temperatures. This effect might be due to increased metabolism at higher temperatures leading to a more rapid depletion of food reserves.

3. Surface area-volume relationships. It has been observed that mating occurs slowly and involves only a few pairs in deep suspensions of washed cells. The same cell mixture will produce abundant conjugating pairs in a much shorter time when placed in shallow containers such as depression slides or watch glasses. The influence of fluid depth on the refractory period is illustrated by the following experiment.

Ten ml. of a distilled water suspension of thoroughly washed cells (types I and II) were placed in test tubes in which the fluid depth was 80 mm. and in small beakers in which the fluid depth was 8 mm. at 22° C. The refractory period was prolonged from 5–7 hours in the beakers to 22–28 hours in the test tubes.

Anoxia is possibly the reason for failure to obtain conjugation in deep containers. Aeration of deep suspensions by bubbling air through the fluid failed to speed up mating but this failure may have been secondarily induced by agitation alone.

4. *Agitation.* Mechanical agitation (shaking, stirring, etc.) of a mixture, which under quiet conditions mated normally, prevented conjugation completely. Disturbing the protozoa during mating by drawing them into and forcing them out of a pipette also stopped conjugation. It appears that they must remain relatively quiet if mating is to be successful.

5. *Age of culture.* There are visible differences between young (2–4 day) stock medium cultures and those a week or more old. During the first 4 or 5 days of incubation all organisms in the tube are suspended throughout the medium with heavier concentrations in the upper levels. Longer periods of incubation result in a gradual accumulation in the bottom of the tube of debris composed of cellular fragments and many swollen, distorted and sluggish organisms. After a month there

TABLE II

Effect of temperature on conjugation

Temperature ° C.	
1–2°	No conjugation, cells spherical and motionless
23–25°	Conjugation in 6–8 hours
27–28°	Conjugation in 3–5 hours
31–32°	Conjugation in 3–4 hours
37–38°	Conjugation in 3 hours
40–42°	No conjugation
46–49°	No conjugation, cells dead in 2 hours

are very few active "normal" individuals. The question as to whether or not there was any correlation between this change in appearance and activity induced by age, and the refractory period was answered in the following manner. A series of (type I and II) stock medium cultures of varying ages (3, 5, 7, 11, 13, 35 days) was centrifuged and washed through four changes of distilled water. The organisms were then mixed in shallow depression slides and examined at intervals to record the appearance of the first mating pairs. In all cases the activation period was 2–4 hours in length. Age of the culture, within these limits, has no influence on the length of the refractory period.

6. *Sexual immaturity.* All exconjugant clones of *Tetrahymena* established thus far show a temporary loss of mating type. For a certain length of time after isolation the young clone does not conjugate with other clones. This is believed to be the same period of sexual immaturity reported for some varieties of *Paramecium* and other conjugating ciliates (Sonneborn, 1939).

A total of 52 exconjugants from various matings has been isolated and maintained as clone cultures. All of these failed to conjugate with parental types I, II, or III, or with each other, for two to four weeks after isolation. Serial transfers had no influence on the duration of this period. In several cases, where tested, first trans-

fer cultures regained mating ability at the same time as fourth, fifth and sixth transfers.

It is believed that length of the immaturity period is determined by fission rate, *viz.*, the exconjugant must undergo a certain number of cell divisions before the clone becomes sexually mature as observed by Maupas (1889) in *Tetrahymena patula*. If this is correct, cultural conditions which speed up the fission rate should correspondingly shorten the immaturity period.

Cell division and population growth are influenced within certain limits by temperature. Initial growth is faster at 32° C. than at 22° C. Also, flask cultures with larger surface areas develop heavier populations than tube cultures (Elliott *et al.,* 1952). To determine the effect of faster growth on the time required to reach maturity, parallel series of flasks and tubes of stock medium were inoculated from recently isolated immature exconjugant clones. Sets of cultures were incubated at 22° C., 25° C., and 32° C., and at daily intervals cells were removed, washed, and tested to determine their ability to conjugate with types I, II, and III. At 32° C. both flask and tube cultures did not conjugate until the 18th day after isolation, at 25° C. both flask and tube cultures matured on the 19th day. Those cultures incubated at 22° C. did not mature until the 26th day. Although these results are not conclusive, they indicate that the length of the immaturity period is correlated, in part, with fission rate. Further experiments are planned to demonstrate this correlation more precisely and to develop culture techniques which will shorten the maturation time as much as possible.

Discussion

At intervals during the long period that *Tetrahymena pyriformis,* strain E, has been grown in axenic cultures (since 1932), attempts were made to induce conjugation with other strains with the hope that, should this be possible, its genetic system might be studied. Unfortunately, conjugation was never observed in any of the long established strains (E, W, GL, H) when mixed in all combinations (among themselves and with mating types I, II, and III) and under conditions which normally induce conjugation in other ciliates. Failure may be attributed to the fact that none of them possesses micronuclei. However, amicronucleate ciliates have been known to conjugate. Schwartz (1939) observed mating in *Paramecium bursaria* between experimentally enucleated individuals and normal cells. Diller (1936) reported conjugation between micronucleate and amicronucleate races of *P. aurelia*. From this evidence it seems possible that conjugation can be obtained in some of the many amicronucleate strains of *T. pyriformis* by mixing them with micronucleate strains although this has never been observed in several hundred trials. However, information reported here seems to point to the fact that there exist in nature other mating types and other varieties of *T. pyriformis*. If and when these are found, some of the problems concerning genetics and the significance of amicronucleate races may be solved.

Long ago Maupas (1889) described the cytological details of conjugation in a number of ciliates, among them *"Leucophrys patula,"* a species recently transferred to the genus *Tetrahymena* (Corliss, 1952b, 1953). More recently Horn (1951) observed mating in a strain of *Tetrahymena pyriformis*. Corliss (unpublished work) has studied conjugation in strains of *Tetrahymena* [*Glaucoma*]

parasitica (personal communication). All of these workers were using bacterized cultures and they observed only selfing in which no exconjugants survived. The strains of selfers isolated from the Ann Arbor region (AA strains) mate readily under axenic conditions but they, too, fail to survive the process. The widespread occurrence of a sexual phenomenon so deleterious to the survival of a species as selfing seems to be, at least as it occurs under laboratory conditions, stimulated a search for conjugating strains with surviving progeny. Assuming that conjugation is beneficial to a species it is reasonable to expect that somewhere in nature mating types of *Tetrahymena* exist. The success of this search was enhanced by the fact that the mating types described here do conjugate readily under axenic conditions. Had this not been the case any genetic studies involving nutrition would have been greatly handicapped if not impossible.

It is well known that nutritional conditions of the medium are important in the sexual behavior of ciliates. For example, Chatton and Chatton (1929a) found that *Glaucoma scintillans* underwent conjugation only in the presence of certain bacterial metabolic products while Seckbach (1948) found that bacteria were unnecessary when rye grain, liver, and intestinal mucosa extracts were used. In the present investigation it is possible to control the nutrition in all respects which emphasizes the usefulness of *Tetrahymena* for genetic studies.

The nutrient requirements, insofar as the amino acids and B-vitamins are concerned, are identical for the three mating types and strain E. This is not surprising in view of the remarkable consistency in the requirements of the long established strains. Kidder and Dewey (1945) were able to show some differences in carbohydrate fermentation among several strains of *Tetrahymena pyriformis* (H, E, T-P, T, W, GHH) and *T. vorax* (V_2, PP). With the exception of strains W and GHH, which grew without serine, all required the eleven amino acids that are also essential for the mating types. Likewise, the vitamin requirements of the mating types correlate closely with that of the long established strains. Until more information is available for other closely related ciliates it is impossible to conjecture how universal these requirements are.

The conjugation process in *Tetrahymena* agrees with the generalized sequence of nuclear and cytoplasmic events established for other ciliates. It involves a temporary pairing of two animals, three prezygotic divisions of the micronucleus producing haploid gametic nuclei in each conjugant, mutual exchange of gametic nuclei and their union to form a synkaryon, three postzygotic divisions of the synkaryon, and subsequent reorganization stages of the exconjugants. The most significant difference between the mating types of *Tetrahymena* and the "selfers" described by Maupas (1889), Horn (1951) and Nanney (1953) lies in the fact that viable exconjugant clones are produced. Selfing seems to be a lethal process, whereas exconjugants from the Woods Hole strain undergo a third postzygotic division of the functional micronucleus and divide to produce viable progeny.

In reviewing the literature concerning conjugation and the mating reactions of ciliates one is impressed in many cases with the extreme periodicity or irregularity of the process. Whether or not conjugation takes place when two appropriate cultures are mixed seems to depend on a number of exacting environmental and physiological conditions. The induction of conjugation in *Tetrahymena* seems remarkably easy and simple by contrast.

Wichterman (1953) reported that time of day had a pronounced influence on

the mating reaction in *Paramecium bursaria*. With a few exceptions mating does not occur in the early morning hours or after 5 or 6 P.M., and the reaction is greatest at 12 o'clock noon. Similar diurnal periodicities were found by Jennings (1939) in his varieties of *P. bursaria* and by Sonneborn (1939) in some varieties of *P. aurelia*. Conjugation has been obtained in *Tetrahymena* at any time of day or night. There is no evidence of a diurnal periodicity of any sort, or of sexual inhibition by light or darkness.

The effect of temperature on conjugation has also been investigated by several workers. Sonneborn (1939) found striking varietal differences in this regard with *P. aurelia*. Although mating types of variety 1 will conjugate at any temperature within the range examined, 9° C. to 32° C., mating types of variety 2 will not react above 24° C., and types of variety 3 not above 27° C. Strong mating reactions in *P. caudatum* at temperatures ranging from 18° C. to 24° C. were reported by Gilman (1939). Giese (1939) in a study of temperature effects on conjugation in *P. multimicronucleatum* observed that animals grown at 30° C. seldom were found to mate but did so when placed at lower temperatures. *Tetrahymena* conjugates readily at all temperatures within the range 17° C. to 38° C.; temperatures below 17° C. have not been investigated thoroughly. The principal influence of temperature seems to be on the "refractory period." Mating takes place sooner at 38° C. than at lower ranges.

There are scattered reports in the literature of the influence of certain chemical factors and pH on conjugation in ciliates. For example Sonneborn (1939) reported that the mating reaction in *P. aurelia* is weak or lacking when "deleterious bacteria," or presumably, metabolic products of these bacteria, injure the paramecium. Chatton and Chatton (1929a) studied a variety of chemical compounds, including $CaCl_2$, $FeCl_3$, pyruvic acid, glucose, and bacterial metabolic products as "zygogenic agents" influential in initiating conjugation of *Glaucoma scintillans*. The zygogenic effects of these compounds were re-investigated by Seckbach (1948) who added salts of barium and magnesium to the list of factors essential or stimulatory for conjugation in this species. Wichterman (1953) found that pH within the range 6.0 to 8.0 had little influence on conjugation of *P. multimicronucleatum*. Since we have been able to obtain conjugation so readily in *Tetrahymena* simply by mixing washed mating types in distilled water a search for specific chemical factors has not been made. The pH of unbuffered water suspensions in which conjugating pairs are found varies from 6.6 to 7.7.

The nutritive state of the protozoan seems to be an important factor influencing conjugation in all ciliates. Maupas (1889) lists hunger, sexual maturity, and diverse ancestry as the three conditions necessary for mating in ciliates. Sonneborn (1939) observed that in *P. aurelia* the mating reaction does not take place in cultures that are either over-fed or completely starved. A similar situation exists in *P. caudatum* (Gilman, 1939) and *P. bursaria* and *P. calkinsi* (Wichterman, 1953). Giese (1939) found that food appeared to be the most important single factor in regard to conjugation in *P. multimicronucleatum* and that a decline in available food after a period of plenty was required. Chatton and Chatton (1929b) stated that starvation was a necessary condition for conjugation of *Glaucoma scintillans*. Evidence obtained thus far on the effect of starvation on conjugation of *Tetrahymena* agrees in general with that reported for other ciliates.

Tetrahymena must be at least partially starved before it will conjugate. Unlike those ciliates which seem to require a nutritional state intermediate between well-fed and completely starved, *Tetrahymena* has been found to conjugate normally even after starving in distilled water for 3 or 4 days.

One of the three conditions necessary for conjugation in ciliates, as cited by Maupas, is sexual maturity. Observations by many investigators indicate that in some species, at least, conjugation can be induced only when the animals are sexually mature. According to Calkins (1933) *Uroleptus mobilis* will mate only after a period of from 5 to 10 days following fertilization. Many races of *P. aurelia* (Sonneborn, 1939) do not conjugate during the first week or two; other races, however, lack this period of immaturity and are able to mate again immediately following conjugation. Jennings (1939) reported a state of sexual immaturity for *P. bursaria* ranging from a few weeks to several months. Periods of immaturity have been found in *P. caudatum* by Gilman (1939) and in *Euplotes* by Kimball (1939). *Tetrahymena* also goes through a definite period of immaturity during which exconjugants are not sexually reactive for a week or more. It may be possible to correlate this period with number of fissions.

From the observations reported here it seems quite clear that *Tetrahymena* has potentialities as a tool for further investigations in protozoan genetics and should supplement the already voluminous literature on *Paramecium* and other ciliates. Moreover, because its biochemistry and physiology are well known, it should become an important addition to the list of microorganisms that have already greatly enhanced our knowledge of biochemical genetics.

Summary

1. Conjugating strains representing three different mating types of *Tetrahymena pyriformis* have been isolated from fresh water ponds in the Woods Hole, Massachusetts, area and established in axenic cultures.

2. These strains have the same amino acid and vitamin requirements as *T. pyriformis E*.

3. The cytology of conjugation is described.

4. Routine laboratory procedures for obtaining conjugation and establishing exconjugant clones (F_1 and successive generations) are presented.

5. Some of the factors influencing conjugation in *T. pyriformis* (nutritive state, temperature, oxygenation, agitation, culture age, and sexual maturity) are discussed and compared with reports in the literature dealing with conjugation in other ciliates.

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